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(54) ENZYME TREATMENT OF GLUCANS

BEHANDLUNG VON GLUCANEN MIT ENZYMEN

TRAITEMENT ENZYMATIQUE DES GLUCANES

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- **FEBS LETTERS, vol. 64, no. 1, 1976 pages 44-47, S.BALINT ET AL. 'Biosynthesis of Beta-Glucans catalyzed by a particulate enzyme preparation from yeast'**
- **J. BIOCHEM., vol. 98, no. 5, 1985 pages 1301-1307, MASAO SHIOTA ET AL. 'Comparison of Beta-Glucan structures in a cell wall mutant of Sacch. cerevisiae and the wild type'**
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Description

[0001] This invention relates to structurally modified yeast glucans, especially but not exclusively from the family Saccharomyces, obtainable by using β-(1-6)-glucanase. The use of such modified glucans in vaccine and animal feed formulations is described

Background of the Invention

[0002] It is known from European Patent Application No. 91111-143.3 (Publication No. 0466037 A2) that the immune system of aquatic animals can be stimulated through the administering of an effective amount of a yeast cell wall glucan. It is further known that the effect of vaccines on such aquatic animals can be enhanced by the administering of an effective amount of such yeast cell wall glucan along with the vaccine antigens.

[0003] Such glucan compositions are particulate glucans such as that derived from the yeast Saccharomyces cerevisiae. Such particulate glucans are macromolecular and are comprised of a chain of glucose units linked by β-(1-3)- and β-(1-6)-linkages, said glucan being a branced β-(1,3)-glucan having β-(1,3)-linked and β-(1,6)-linked chains therein.

[0004] Such particulate glucans are provided by KS Biotec-Mackzymal under the brand "MacroGard" and are potent activators of the macrophage/monocyte cell series. Thus such particulate glucans have a profound effect on the immune system.

[0005] While the particulate glucan derived from Saccharomyces cerevisiae is recognized to have a variety of beneficial effects on fish and other animals, the use of the glucan in the particulate and thus insoluble form is limited.

[0006] In addition it is now believed that the presence of β-(1-3)-branches contribute to the desired pharmaceutical benefits to be obtained from particulate glucan.

[0007] Accordingly a system whereby the of β-(1-3)-linked branches are made more readily available in the glucan would be highly desirable.

[0008] U.S. Patent No. 5,028,703 discloses and claims whole glucan particles wherein the β-(1-6) and β-(1-3)-linkage have been altered. Database WPI, Section ch, week 7949 class b04, AN 79-88295b discloses what is believed to be a glucan comprising mainly β-(1-3)-glucoside bonds since it is decomposed by β-(1-3)-glucanase.

[0009] Publication entitled *Specificity of Beta-glucan Receptor on Macrophages from Atlantic Salmon* from the Development and Comparative Immunology, vol. 18, no. 5, pp 397-408, 1994 provides an overview of a study of beta-glucan receptors in Atlantic salmon macrophages.

[0010] FEBS Letters, vol. 64, no. 1, 1976, pp 44-47 discloses the biosynthesis of β-glucans catalyzed by a particulate enzyme preparation of yeast.

SUMMARY OF THE INVENTION

[0011] It has been discovered that by treating the particulate glucan derived from yeast organisms, especially of the family Saccharomyces, and particularly Saccharomyces cerevisiae, with a β-(1-6)-glucanase, there is obtained a modified particulate glucan which is characterized by its enhanced activity in effecting stimulation of the immune system.

[0012] Thus in one embodiment of the present invention there is provided a novel β-(1-3)-glucan derived from yeast as defined in claims 1 and 5. As noted above, the yeast glucan is characterized by its enhanced ability to stimulate the immune system of fish and other animals.

[0013] In another embodiment of this invention there is provided a novel solubilized β-(1-3)-glucan from yeast which is useful for enhancing the activity of veterinary vaccines.

[0014] In still another embodiment of the present invention there is provided a novel feed grade glucan composition which is useful as an ingredient in conventional animal feeds.

[0015] Other embodiments and advantages of this invention will be apparent from the following specifications and claims.

Process for preparation of β-(1-6)-glucanase treated glucan ("MacroGard").

[0016] "MacroGard" brand glucan is derived from Saccharomyces cerevisiae as disclosed in European Application No. 91111143.3. While such glucan is known to stimulate the immune system of fish, it has been discovered that its activity is enhanced by the treatment thereof with a β-(1-6)-glucanase.

[0017] Such glucanase treatment of the glucan is carried out by suspending the glucan particle in a buffered medium at a pH in the range of about 4 to about 8 and at a temperature in the range of from about 20 to about 50°C. Suitable buffered media are those selected from the group consisting of sodium acetate, ammonium acetate and sodium-potassium phosphate. Presently preferred buffer solutions are sodium acetate or ammonium acetate. Enzymatic degradation of the glucan is commenced by the addition of the β-(1-6)-glucanase to the buffered medium.

[0018] β-(1-6)-glucanases which are suitable for the modification of yeast glucan are those obtained from a microorganism selected from the group consisting of Trichoderma longibrachiatum, Trichoderma reesei, Trichoderma harzianum, Rhizopus chinensis, Gibberella fujikuroi, Bacillus circulans, Mucor lilmalis, and Acineto-bacter. Of these a presently preferred glucanase is that obtained from Trichoderma harzianum.

[0019] The amount of β-(1-6)-glucanase employed for treatment of the glucan is normally in the range of from 1 to 50 U per g of glucan.

[0020] Enzymatic degradation is terminated by heating the reaction mixture to a temperature in the range of 80 to 100°C, preferably for a time in the range of 2 to 10 min. Other ways to stop the enzyme degradation are, e.g. by adding proteases or inhibitors to the reaction mixture.

[0021] Alternatively the enzyme may be simply removed by washing. The washed particles are then resuspended in water with the addition of a bactericide such as 0.3% formalin (v/v) and stored at a temperature of about 4°C.

[0022] The resulting enzyme treated glucan can be characterized as a branched β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being essentially free of β-(1-6)-linked chains. In this connection the phraze "β(1-6) chains" is meant to include branches of more than 1 β(1-6)-linked glucose units. The β-(1-6)-glucanase enzyme cleavage ensures that most chains of more than 4 β-(1-6)-bound glucose units are cleaved off.

[0023] To further enhance the utility of the glucan, it is subject to solubilization. Such solubilization treatment is generally carried out at a temperature in the range of about from 70 to 90°C for a period of from about 30 to 60 min. in the presence of a solubilizing agent. A presently preferred solubilizing agent is formic acid. Following solubilization the solubilizing agent is removed and the resulting glucan is boiled in distilled water.

[0024] The glucan can be first enzyme treated and then solubilized or conversely be solubilized and then enzyme treated.

[0025] In accordance with another embodiment of this invention there is provided a β-(1-6)-glucanase treated feed grade glucan from yeast, e.g. Saccharomyces cerevisiae. Such feed grade glucan can be obtained by first contacting the yeast cell wall with an aqueous alkaline solution under conditions to effect the extraction of proteins and lipids therefrom. Generally such extraction is carried out at a temperature in the range from about 50 to 80°C for about 2 to 8 h. A presently preferred alkaline extraction agent is sodium hydroxide. Following extraction, the cell walls are recovered from the aqeous alkaline solution and washed to remove solubilized cell wall components therefrom. The washed yeast cell wall are then neutralized by treatment with an acid such as phosphoric acid. Thereafter the neutralized washed glucan is pasteurized and then dried.

[0026] Suitable enzymes for treatment of the feed grade glucan are those useful in treating the high purity glucan.

[0027] The enzyme treated feed grade glucan is prepared by contacting the glucan with a β-(1-6)-glucanase in the same manner as that employed for the enzyme treatment of the glucan particulate. The β-(1-6)-glucanase treated feed grade glucan of this invention is useful in animal feed formulations.

[0028] The following examples are presented for purposes of illustration of the invention.

EXAMPLE 1

[0029] This example provides the protocol used to obtain an immunostimulatory glucan particle suitable for utilization in the preparation of the yeast glucan of the present invention.

[0030] 500 g of dry Saccharomyces cerevisiae was suspended in 3 l of 6 % aqueous NaOH solution. This suspension was then stirred overnight at room temperature. After stirring the suspension was centrifuged at 2000 x g for 25 min. The supernatant was discarded and the insoluble residue was then resuspended in 3 l of 3 % NaOH and incubated for 3 h at 75°C followed by cooling the suspension overnight. The suspension was then centrifuged at 2000 x g for 25 min. and the supernatant was decanted. The residue was then resuspended in 3 % NaOH, heated and centrifuged as previously described.

[0031] The insoluble residue remaining was then adjusted to pH 4.5 with acetic acid. The insoluble residue was then washed with 2 l of water three times and recovered by centrifuging at 2000 x g for 25 min after each wash (the supernatant was poured off). The residue was then suspended in 3 l of a 0.5 M aqueous acetic acid. The suspension was heated for 3 h at 90°C. The suspension was then cooled to room temperature. After cooling, the insoluble residue was then collected by centrifuging at 2000 x g for 25 min. This treatment (from adjusting to pH 4.5 to collecting the cooled residue) was repeated 6 times.

[0032] The insoluble residue was then suspended in 3 l of distilled water and stirred for 30 min at 100°C, then cooled and centrifuged at 2000 x g for 25 min. The supernatant was discarded. The insoluble residue was washed in this manner 4 times. The residue was next suspended in 2 l of ethanol and heated at 78°C for 2 h. This wash with ethanol was repeated 4 times. The residue was then washed 4 times with 3 l of distilled water at room temperature to remove the ethanol, thereby providing a suspension of desired glucan product.

[0033] In more specific terms, the stepwise process for the production of the particulate β-(1-3)-glucan entails (a) alkali-extracting suitable glucan-containing yeast cells with a suitable extractive aqueous alkali solution under suitable

conditions to provide a first insoluble yeast residue; (b) hot alkali-extracting said first insoluble yeast residue with a suitable extractive aqueous alkali solution under suitable extraction conditions wherein the hot alkali extraction is performed at least 2 times to provide a second insoluble yeast residue and recovering the insoluble yeast residue after hot alkali extraction; thereafter (c) washing said second insoluble yeast residue with a suitable hydrolyzing acid under suitable conditions with water at a pH in the range of from about pH 4 to about pH 7 thereby providing a third insoluble yeast residue and recovering said third insoluble yeast residue after the wash; (d) hydrolyzing said third insoluble yeast residue under mild acidic hydrolysis condition wherein the acid hydrolysis is performed at lest 3 times to provide a fourth insoluble yeast residue and recovering the yeast residue after each hydrolysis; thereafter (e) boiling said fourth insoluble yeast residue under suitable conditions in water wherein the boiling of said fourth insoluble yeast residue is performed at least 2 times to provide a fifth insoluble °yeast residue and recovering the insoluble yeast residue after each boiling; and (f) boiling said fifth insoluble yeast residue under suitable conditions in ethanol wherein the boiling in ethanol of said fifth yeast residue is performed at least 2 times to provide a sixth insoluble yeast residue and recovering the insoluble yeast residue after each boiling; thereafter (g) washing said sixth insoluble yeast residue under suitable conditions with water wherein the washing of said sixth yeast residue is performed at least 2 times to provide a yeast glucan and recovering the insoluble yeast residue after each wash.

EXAMPLE 2

[0034] This example provides the protocol to obtain glucan particles essentially free of β-(1-6)-linked chains with the use of β-(1-6)-glucanase isolated from Trichoderma harzianum.

[0035] 200 mg of glucan particles prepared in accordance with the Example 1 were suspended in 40 ml 50 mM ammonium acetate buffer, pH 5.0, together with 10 U of β-(1-6)-glucanase at 37°C for 6h with constant stirring. The enzymatic degradation of the glucan particles was ended by heating the suspension at 100°C for 5 min. The particles were then washed three times with 200 ml sterile distilled H_2O by centrifugation at 2000 x g for 10 min, whereafter 185 mg of dried enzyme treated glucan was obtained.

[0036] The enzyme treatment will only cleave β-(1-6)-linkages within β-(1-6)-linked chains, but will not remove the β-(1-6)-linked glucosyl residue extending from the branching points. The resulting enzyme treated glucan can be characterized as a branched β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being essentially free of β-(1-6)-linked chains, i.e. being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound glucose units.

EXAMPLE 3

[0037] This example provides the protocol to solubilize glucan particles prepared in accordance with Example 1 by hydrolysis using formic acid (HCOOH).

[0038] 2.0 g of glucan particles were suspended in 1.0 l of 90% formic acid and heated at 80°C for 45 min under constant stirring. The suspension was cooled to 35°C and the formic acid was evaporated. The residue containing the hydrolysed particles was boiled in 500 ml distilled water for 3 h, whereafter the cooled suspension was filtrated through a 0.44 µm filter, frozen and lyophilized whereby 1.9 g of dry solubilized particles were obtained. The lyophilized solubilized particles were then dissolved in 100 ml distilled water and dialyzed, using a tubular dialysis membrane having a nominal molecular weight cut off (NMWCO) of 5000 Dalton, against tap water for 24 h, and then lyophilized. This resulted in 1.8 g solubilized glucan product.

EXAMPLE 4

[0039] This example demonstrates the biological effects of glucan particles prepared according to Example 1, and β-(1-6)-glucanase treated glucan particles prepared according to Example 2 on immune responses in Atlantic salmon.

[0040] An A-layer positive isolate of Aeromonas salmonicida subsp. salmonicida, referred to as strain no. 3175/88 (Vikan Veterinary Fish Research Station, Namsos, Norway) was used. The bacterium was grown in brain heart infusion broth (Difco, USA) for 30 h at 14°C in a shaker incubator, and the culture medium with the bacterium was centrifuged for 10 min at 3000 x g. The pellet was resuspended in 0.9% saline, and the bacterium was killed by adding 0.5% formalin (v/v) to the suspension and incubating at 14°C for 24 h. The formalinized culture was then washed with sterile 0.9% saline and resuspended to a concentration of 2×10^9 ml^{-1} bacteria in 0.9% saline with 0.3% formalin. Bacterial suspensions ware mixed with an equal volume of saline or with the different glucan suspensions (10 mg ml^{-1} in saline). Formalin was added to the vaccines to a final concentration of 0.3% (v/v).

[0041] In carrying out these experiments, two groups of experimental fish were used. In the vaccine experiment, Atlantic salmon presmolts of 20 - 40 g were used. In the experiment where serum was collected for measuring blood lysozyme activity after glucan injection, Atlantic salmons of 50 - 70 g were used. The fish were kept in 150 l tanks

supplied with aerated fresh water at 12°C and fed with commercial pellets ad libitum twice daily.

[0042] In the vaccination experiment 40 fish in each group were IP-injected with 0.1 ml of the different vaccine preparations or vaccine without glucan as a control. Blood was collected in evacuated tubes (Venoject, Terumo-Europe, Belgium) from 10 fish in each group 6, 10, and 18 weeks after injection. Blood samples were allowed to clot overnight at 4°C and sera were collected after centrifuging the tubes at 2000 x g for 10 min. Individual serum samples were transferred to Micronic serum tubes (Flow Laboratories Ltd., Lugano, Switzerland) and stored at -80°C until required.

[0043] In order to measure the effect of glucans on blood lysozyme activity, salmons were IP injected with 0.3 ml of the different glucans in saline or with 0.3 ml saline as the negative control. The glucans were administered at a concentration of 10 mg ml^{-1}. Blood samples were collected from 10 fish from each group 10 and 20 days after injection, using evacuated tubes (Venoject). The tubes were kept on ice until centrifuged at 2000 x g for 10 min, and individual serum samples were transferred to Micronic serum tubes and stored at -80°C until required.

[0044] Lysozyme activity was measured with the turbidimetric method using 0.2 mg ml^{-1} lyophilized Micrococcus lysodeikticus as the substrate in 0.04 M sodium phosphate buffer at pH 5.75. Serum (20 µl) was added to 3 ml of the suspension and the reduction in absorbance at 540 nm was measured after 0.5 min and 4.5 min at 22°C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. Results are expressed as mean lysozyme activity in serum from 10 fish (Tables 1 and 2).

[0045] The level of specific antibody against the A-layer of A. salmo-nicida in salmon sera was measured by an enzyme-linked immunosorbent assay (ELISA). A-layer protein was purified from whole A. salmonicida cells (Bjørnsdottir et al. (1992), Journal of Fish Diseases, 15:105-118), and protein content was determined (Bradford, M.M. (1976), Analytical Biochemistry, 72:248-254) using a dye-reagent concentrate from Bio-Rad Laboratories (Richmont, USA). Microtitre plates were coated with 100 µl of 5 µg ml^{-1} A-layer protein in 50 mM carbonate buffer, pH 9.6, and incubated overnight at 4°C. The further procedure was performed as described by Havardstein et al. (Journal of Fish Diseases (1990), 13:101-111). The antibody titre in pooled serum samples was determined before individual serum samples were measured at three different dilutions (1:500, 1:1000 and 1:2000). Absorbance was read at 492 nm in a Multiscan MCC/340 MK II (Flow Laboratories Ltd). Results are expressed as mean antibody response to the A-layer of the bacterium at a dilution of 1:2000 in serum from 10 fish (Tables 1 and 2).

Table 1.

Differences in biological effects of glucan particles and β-(1-6)-glucanase treated glucan particles on immune responses in Atlantic salmon.

	Saline control	Untreated glucan particles	β-(1,6)-glucanase treated glucan particles
Lysozyme activity post injection (units/ml)			
10 days	304	505	529
20 days	330	407	454
	Vaccine without glucan	Vaccine with untreated glucan particles	Vaccine with β-(1,6)-glucanase treated glucan particles
Antibody response post injection (absorbance)			
6 weeks	0.165	0.255	0.376
10 weeks	0.059	0.355	0.500
18 weeks	0.037	0.197	0.142

[0046] Both injection of untreated and β-(1,6)-glucanase treated glucan particles induced significantly higher ($p<0.01$) lysozyme activity in serum compared to saline control both 10 and 20 days post injection. At day 20 post injection the lysozyme levels in fish injected with β-(1,6)-glucanase treated glucan particles were significantly higher ($p<0.05$) compared to fish injected with untreated particles.

[0047] β-(1,6)-glucanase treated glucan particles induced significantly higher ($p<0.05$) antibody response to the vaccine compared to vaccine without adjuvant at all three sampling times, whereas untreated glucan particles induced significantly higher response 10 and 18 weeks post injection. β-(1,6)-glucanase treated glucan particles induced significantly higher ($p<0.05$) antibody response than did untreated glucan particles at 10 weeks post injection, whereas no significant differences between the two were observed at 6 and 18 weeks post injection.

Table 2.

Biological effects of glucan particles and solubilized glucan.			
	Saline control	Untreated glucan particles	Solubilized glucan particles
Lysozyme activity (units/ml)			
10 days after injection	304	505	603
20 days after injection	330	407	773
	Vaccine without glucan	Vaccine with untreated glucan particles	Vaccine with solubilized glucan particles
Adjuvant effect (absorbance)			
6 weeks after injection	0.165	0.255	0.184
10 weeks after injection	0.059	0.355	0.349
18 weeks after injection	0.037	0.197	0.120

[0048] Injection of solubilized glucan particles induced significant higher ($p<0.01$) lysozyme activity than did untreated glucan particles both 10 and 20 days post injection. No significant differences could be observed between the ability of solubilized glucan particles and untreated glucan particles to induce increased antibody response to the vaccine antigen at any sampling time point. Both induced significant higher ($p<0.05$) antibody response than vaccine without adjuvant 10 and 18 weeks post injection, but not at 6 weeks post injection.

EXAMPLE 5

[0049] This example provides the protocol to obtain a glucan composition suitable for use in the feeding of animals.

[0050] 1000 kg of dry cell wall material of Saccharomyces cerevisiae was suspended in 5300 l of water at a temperature of 65°C in a stainless steel tank. To the suspension of cell walls in water there was added 227 l of 50% w/w NaOH so as to provide a caustic concentration of about 3%. The resulting mixture was then stirred for a period of about 4 h at a temperature of about 60°C.

[0051] Following the initial extraction period the suspension was then diluted with 8000 kg of water at a temperature of about 65°C in a stainless steel, stirred, washing tank such that the weight of the mixture was doubled. The resulting diluted mixture was then maintained at a temperature of about 60°C while being stirred for a period of about 15 min. Thereafter the resulting mixed diluted suspension was centrifuged in a nozzle centrifuge (Alfa Laval DX209). The supernatant was discarded. The resulting concentrated cell wall suspension was continuously introduced into a second steel stirred wash tank containing 8000 kg water and the mixture adjusted by the addition of water to give a final weight of 14500 kg. The resulting suspension was then mixed for a period of 15 min at a temperature of 60-65°C. Thereafter the agitated mixture was centrifuged.

[0052] The resulting cell wall suspension was continuously added to a third vessel containing 8000 kg water. Additional water at 60°C was added to provide a final weight of 14500 kg. The resulting suspension was stirred for a period of 15 min at 60-65°C and thereafter centrifuged.

[0053] Following centrifugation, the resulting cell wall concentrate was transferred to a stainless steel storage tank wherein the suspension was cooled to a temperature of about 5-10°C. The resulting cooled suspension was treated with phosphoric acid (H_3PO_4) in a stainless steel agitated tank in an amount to achieve a suspension of solids having a pH of 5.5-7.5.

[0054] Following neutralization the resulting neutralized mixture was subjected to pasteurization by heating at a temperature of 75°C for a period of 18 seconds by passing the mixture through an in-line plate and frame heat exchanger.

[0055] Following pasteurization the resulting pasteurized mixture was then spray dried in a spray drier maintained at an inlet air temperature of at least 140-150°C and an exhaust temperature of about 65-70°C whereby there was achieved 300 kg of dry glucan product.

EXAMPLE 6

[0056] This example provides the protocol and effect of treatment of feed grade glucan with a β-(1-6)-glucanase.

[0057] 25 g of feed grade glucan, prepared in accordance with Example 5, suspended in 1.25 l of 50 mM sodium acetate, pH 5.0, in a 2 l conical flask. Glucan particles were maintained in suspension by shaking, the suspension was warmed to 30°C and purified β-(1-6)-glucanase from Trichoderma harzianum was added to a final concentration of 1.8 U/g glucan.

[0058] To follow the timecourse of the enzymatic removal of β-1,6-bound glucose from the glucan particle, the suspension were withdrawn at different timepoints, centrifuged at 2000 x g, and 0.2 ml of the supernatants analyzed for free, reducing carbohydrate (Nelson et al. (1944), Journal of Biological Chemistry, (153:315-80). The glucan suspension was incubated for 28h at which time the rate of release of free, reducing carbohydrate was observed to be very low. The glucan particles were then pelleted by centrifugation at 2000 x g, washed once in 50 mM sodium-acetate, pH 5.0 and once in water.

[0059] A fine, dry powder suitable for use as a feed additive was prepared from the wet glucan by first dehydrating the pellet four times with ethanol at room temperature followed by air drying at room temperature.

[0060] The stepwise process for the production of the feed grade glucan includes (a) contacting yeast cell walls with an aqueous alkaline solution under suitable conditions to effect the extraction of proteins and lipids therefrom; (b) separating the resulting extracted yeast cell walls from said aqueous alkaline solution; (c) washing the resulting separate yeast cells so as to further remove solubilized cell wall components therefrom; (d) neutralizing the washed yeast cell walls; and (e) pasteurizing the neutralized, washed cell walls and thereafter drying the resulting pasteurized, neutralized, washed cell walls.

[0061] Results from treating a feed grade glucan with β-(1-6)-glucanase from T. harzianum as described above are shown in Table 3.

Table 3.

Liberation of glucose from feed grade glucan during treatment with β-(1-6)-glucanase from T. harzianum.	
Enzyme reaction time, [h]	Glucose liberated, [% of total glucose in glucan]
0	0.0
0.5	1.9
1	2.6
2	3.3
3	3.7
4	4.0
5	4.3
2	5.5
8	5.6

Claims

1. A branched β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound gluclose units, said glucan being obtainable by the process of contacting an insoluble, particulate, branched β-(1-3)-glucan derived from yeast having β-(1-3)-linked and β-(1-6)-linked chains therein with a β-(1-6)-glucanase.

2. The glucan of claim 1, wherein said insoluble, particulate, branched β-(1-3)-glucan having β-(1-3)-linked and β-(1-6)-linked chains therein is prepared by the process comprising:

   (a) alkali-extracting suitable glucan-containing yeast cells with a suitable extractive aqueous alkali solution under suitable conditions to provide a first insoluble yeast residue;
   (b) hot alkali-extracting said first insoluble yeast residue with a suitable extractive aqueous alkali solution under suitable extractive conditions, wherein the hot alkali extraction is performed at least 2 times to provide a second insoluble yeast residue, and recovering the insoluble yeast residue after hot alkali extraction; thereafter
   (c) washing said second insoluble yeast residue with a suitable hydrolyzing acid under suitable conditions with water at a pH in the range of about pH 4 to about pH 7, thereby providing a third insoluble yeast residue, and recovering said third insoluble yeast residue after the wash;
   (d) hydrolyzing said third insoluble yeast residue under mild acidic hydrolysis condition, wherein the acid hydrolysis is performed at least 3 times to provide a fourth insoluble yeast residue, and recovering the yeast residue after each acid hydrolysis; thereafter

(e) boiling said fourth insoluble yeast residue under suitable conditions in water, wherein the boiling of said fourth insoluble yeast residue is performed at least 2 times to provide a fifth insoluble yeast residue, and recovering the insoluble yeast residue after each boiling; and
(f) boiling said fifth insoluble yeast residue under suitable conditions in ethanol, wherein the boiling in ethanol of said fifth yeast residue is performed at least 2 times to provide a sixth insoluble yeast residue, and recovering the insoluble yeast residue after each boiling; thereafter
(g) washing said sixth insoluble yeast residue under suitable conditions with water, wherein the washing of said sixth yeast residue is performed at least 2 times to provide a yeast glucan, and recovering the insoluble yeast residue after each wash.

3. A solublized β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound glucose units, said glucan being obtainable by the process of contacting a branched glucan product of claim 1 or claim 2 with a solubilizing agent.

4. The solubilized glucan product of claim 3, wherein said solubilizing agent is formic acid and said branched glucan is contacted with said solubilizing agent at a temperature in the range of from 70 to 90 °C.

5. An insoluble, particulate yeast glucan, especially from the yeast family Saccharomyces, and particularly from the yeast species Saccharomyces cerevisiae, being **characterized** as a branched β-(1-3)-glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound glucose units.

6. A branched β-(1-3)-feed grade yeast glucan with β-(1-3)-linked sidechains being attached by a β-(1-6)-linkage and being free of β-(1-6)-linked chains apart from those chains of 4 or less β-(1-6)-bound glucose units, said glucan being obtainable by the process of contacting an insoluble, particulate, branched β-(1-3)-feed grade glucan derived from yeast having β-(1-3)-linked and β-(1-6)-linked chains therein with a β-(1-6)-glucanase.

7. The feed grade yeast glucan of claim 6, wherein said insoluble, particulate, branched β-(1-3)-feed grade glucan having β-(1-3)-linked and β-(1-6)-linked chains therein is prepared by the process comprising:

(a) contacting yeast cell walls with an aqueous alkaline solution under suitable conditions to effect the extraction of proteins and lipids therefrom;
(b) separating the resulting extracted yeast cell walls from said aqueous alkaline solution;
(c) washing the resulting separate yeast cells so as to further remove solubilized cell wall components therefrom;
(d) neutralizing the washed yeast cell walls; and
(e) pasteurizing the neutralized, washed cell walls and thereafter drying the resulting pasteurized, neutralized, washed cell walls.

Patentansprüche

1. Verzweigtes β-(1-3)-Glucan mit β-(1-3)-verknüpften Seitenketten, die über eine β-(1-6)-Verknüpfung angelagert sind und die frei sind von β-(1-6)-verknüpften Ketten außer solchen Ketten von 4 oder weniger β-(1-6)-gebundenen Glucose-Einheiten, welches Glucan erhalten werden kann mit Hilfe des Verfahrens des Kontaktierens eines unlöslichen, partikulären verzweigten β-(1-3)-Glucans, deriviert von Hefe, mit β-(1-3)-verknüpften und β-(1-6)-verknüpften Ketten darin, mit einer β-(1-6)-Glucanase.

2. Glucan nach Anspruch 1, bei welchem das unlösliche, partikuläre verzweigte β-(1-3)-Glucan mit β-(1-3)-verknüpften und β-(1-6)-verknüpften Ketten darin hergestellt wird nach dem Verfahren, umfassend:

(a) alkalisches Extrahieren geeigneter, Glucan enthaltender Hefezellen mit einer geeigneten extrahierenden wässrigen Alkalilösung unter geeigneten Bedingungen, um einen ersten unlöslichen Heferückstand zu schaffen;
(b) heißes alkalisches Extrahieren des ersten unlöslichen Heferückstands mit einer geeigneten extrahierenden wässrigen Alkalilösung unter geeigneten Extraktionsbedingungen, wobei das heiße alkalische Extrahieren mindestens zweimal ausgeführt wird, um einen zweiten unlöslichen Heferückstand zu schaffen, und Gewinnen des unlöslichen Heferückstands nach der heißen alkalischen Extraktion; und danach

(c) Waschen des zweiten unlöslichen Heferückstands mit einer geeigneten hydrolysierenden Säure unter geeigneten Bedingungen mit Wasser im Bereich von etwa pH 4 bis etwa pH 7, womit ein dritter unlöslicher Heferückstand geschaffen wird sowie Gewinnen des dritten unlöslichen Heferückstands nach der Wäsche;
(d) Hydrolysieren des dritten unlöslichen Heferückstands unter Bedingungen einer milden sauren Hydrolyse, wobei die saure Hydrolyse mindestens dreimal ausgeführt wird, um einen vierten unlöslichen Heferückstand zu schaffen, und Gewinnen des Heferückstands nach jeder sauren Hydrolyse; und danach
(e) Kochen des vierten unlöslichen Heferückstands unter geeigneten Bedingungen in Wasser, wobei das Kochen des vierten unlöslichen Heferückstands mindestens zweimal ausgeführt wird, um einen fünften unlöslichen Heferückstand zu schaffen, und Gewinnen des unlöslichen Heferückstands nach jedem Kochen; und
(f) Kochen des fünften unlöslichen Heferückstands unter geeigneten Bedingungen in Ethanol, wobei das Kochen des fünften unlöslichen Heferückstands in Ethanol mindestens zweimal ausgeführt wird, um einen sechsten unlöslichen Heferückstand zu schaffen, und Gewinnen des unlöslichen Heferückstands nach jedem Kochen; und danach
(g) Waschen des sechsten unlöslichen Heferückstands unter geeigneten Bedingungen mit Wasser, wobei das Waschen des sechsten unlöslichen Heferückstands mindestens zweimal ausgeführt wird, um ein Hefeglucan zu schaffen, und Gewinnen des unlöslichen Heferückstands nach jeder Wäsche.

3. Solubilisiertes β-(1-3)-Glucan mit β-(1-3)-verknüpften Seitenketten, die über eine β-(1-6)-Verknüpfung angelagert sind und die frei sind von β-(1-6)-verknüpften Ketten außer solchen Ketten von 4 oder weniger β-(1-6)-gebundenen Glucose-Einheiten, welches Glucan erhalten werden kann mit Hilfe des Verfahrens des Kontaktierens eines verzweigten Glucan-Produkts nach Anspruch 1 oder Anspruch 2 mit einem Solubilisierungsmittel.

4. Solubilisiertes Glucan-Produkt nach Anspruch 3, wobei das Solubilisierungsmittel Ameisensäure ist und das verzweigte Glucan mit dem Solubilisierungsmittel bei einer Temperatur im Bereich von 70° bis 90°C kontaktiert wird.

5. Unlösliches, partikuläres Hefeglucan, speziell aus der Hefefamilie Saccharomyces und besonders von der Species Saccharomyces cerevisiae, **gekennzeichnet** als ein verzweigtes β-(1-3)-Glucan mit β-(1-3)-verknüpften Seitenketten, die über eine β-(1-6)-Verknüpfung angelagert sind und die frei sind von β-(1-6)-verknüpften Ketten außer solchen Ketten von 4 oder weniger β-(1-6)-gebundenen Glucose-Einheiten.

6. Verzweigtes β-(1-3)-Hefeglucan mit Futtermittelqualität mit β-(1-3)-verknüpften Seitenketten, die über eine β-(1-6)-Verknüpfung angelagert sind und die frei sind von β-(1-6)-verknüpften Ketten außer solchen Ketten von 4 oder weniger β-(1-6)-gebundenen Glucose-Einheiten, welches Glucan erhalten werden kann mit Hilfe des Verfahrens des Kontaktierens eines unlöslichen, partikulären verzweigten β-(1-3)-Hefeglucans mit Futtermittelqualität, deriviert von Hefe, mit β-(1-3)-verknüpften und β-(1-6)-verknüpften Ketten darin, mit einer β-(1-6)-Glucanase.

7. Hefeglucan mit Futtermittelqualität nach Anspruch 6, bei welchem das unlösliche, partikuläre verzweigte β-(1-3)-Hefeglucan mit Futtermittelqualität mit β-(1-3)-verknüpften und β-(1-6)-verknüpften Ketten darin hergestellt wird mit Hilfe des Verfahrens, umfassend:

(a) Kontaktieren von Hefezellwänden mit einer wässrigen alkalischen Lösung unter geeigneten Bedingungen, um die Extraktion von Proteinen und Lipiden daraus zu bewirken;
(b) Separieren der resultierenden extrahierten Hefezellwände aus der wässrigen alkalischen Lösung;
(c) Waschen der resultierenden separierten Hefezellen, um weitere solubilisierte Zellwand-Komponenten daraus zu entfernen;
(d) Neutralisieren der gewaschenen Hefezellwände; und
(e) Pasteurisieren der neutralisierten, gewaschenen Zellwände und danach Trocknen der resultierenden, pasteurisierten, neutralisierten, gewaschenen Zellwände.

Revendications

1. β-(1-3)-glucane ramifié à chaînes latérales β-(1-3)-liées à fixation par une liaison β-(1-6) et sans chaînes β-(1-6)-liées mis à part les chaînes de 4 ou moins de 4 unités de glucose β-(1-6)-liées, ledit glucane pouvant être obtenu par le procédé de mise en contact d'un β-(1-3)-glucane ramifié particulaire insoluble issu de levure ayant des chaînes β-(1-3)-liées et β-(1-6)-liées avec une β-(1-6)-glucanase.

2. Glucane selon la revendication 1, où ledit β-(1-3)-glucane ramifié particulaire insoluble ayant des chaînes β-(1-3)-

liées et β-(1-6)-liées est préparé par le procédé comprenant :

(a) l'extraction alcaline de cellules de levure contenant des glucanes appropriées avec une solution alcaline aqueuse d'extraction appropriée dans des conditions appropriées pour produire un premier résidu de levure insoluble ;
(b) l'extraction alcaline à chaud dudit premier résidu de levure insoluble avec une solution alcaline aqueuse d'extraction appropriée dans des conditions d'extraction appropriées, où l'extraction alcaline à chaud est réalisée au moins deux fois pour produire un second résidu de levure insoluble, et la récupération du résidu de levure insoluble après l'extraction alcaline à chaud ; puis
(c) le lavage dudit second résidu de levure insoluble avec un acide hydrolysant approprié dans des conditions appropriées avec de l'eau à un pH dans le domaine d'environ pH 4 à environ pH 7, pour produire un troisième résidu de levure insoluble, et la récupération dudit troisième résidu de levure insoluble après le lavage ;
(d) l'hydrolyse dudit troisième résidu de levure insoluble dans des conditions d'hydrolyse acide douce, où l'hydrolyse acide est réalisée au moins trois fois pour produire un quatrième résidu de levure insoluble, et la récupération du résidu de levure après chaque hydrolyse acide ; puis
(e) l'ébullition dudit quatrième résidu de levure insoluble dans des conditions appropriées dans l'eau, où l'ébullition dudit quatrième résidu de levure insoluble est réalisée au moins deux fois pour produire un cinquième résidu de levure insoluble, et la récupération du résidu de levure insoluble après chaque ébullition ; et
(f) l'ébullition dudit cinquième résidu de levure insoluble dans des conditions appropriées dans l'éthanol, où l'ébullition dans l'éthanol dudit cinquième résidu de levure est réalisée au moins deux fois pour produire un sixième résidu de levure insoluble, et la récupération du résidu de levure insoluble après chaque ébullition ; puis
(g) le lavage dudit sixième résidu de levure insoluble dans des conditions appropriées avec de l'eau, où le lavage dudit sixième résidu de levure est réalisé au moins deux fois pour produire un glucane de levure, et la récupération du résidu de levure insoluble après chaque lavage.

3. β-(1-3)-glucane solubilisé à chaînes latérales β-(1-3)-liées à fixation par une liaison β-(1-6) et sans chaînes β-(1-6)-liées mis à part les chaînes de 4 ou moins de 4 unités de glucose β-(1-6)-liées, ledit glucane pouvant être obtenu par le procédé de mise en contact d'un produit de glucane ramifié selon la revendication 1 ou la revendication 2 avec un agent solubilisant.

4. Produit de glucane solubilisé selon la revendication 3, où ledit agent solubilisant est l'acide formique et ledit glucane ramifié est mis en contact avec ledit agent solubilisant à une température dans le domaine de 70 à 90°C.

5. Glucane de levure particulaire insoluble, en particulier issu de la famille de levures Saccharomyces, et en particulier de l'espèce de levure saccharomyces cerevisiae, **caractérisé** comme étant un β-(1-3)-glucane ramifié à chaînes latérales β-(1-3)-liées à fixation par une liaison β-(1-6) et sans chaînes β-(1-6)-liées mis à part les chaînes de 4 ou moins de 4 unités de glucose β-(1-6)-liées.

6. β-(1-3)-glucane ramifié de levure de qualité alimentaire à chaînes latérales β-(1-3)-liées à fixation par une liaison β-(1-6) et sans chaînes β-(1-6)-liées mis à part les chaînes de 4 ou moins de 4 unités de glucose β-(1-6)-liées, ledit glucane pouvant être obtenu par le procédé de mise en contact d'un β-(1-3)-glucane ramifié particulaire insoluble de qualité alimentaire issu de levures ayant des chaînes β-(1-3)-liées et β-(1-6)-liées avec une β-(1-6)-glucanase.

7. Glucane de levure de qualité alimentaire selon la revendication 6, où ledit β-(1-3)-glucane ramifié particulaire insoluble de qualité alimentaire ayant des chaînes β-(1-3)-liées et β-(1-6)-liées est préparé par le procédé comprenant:

(a) la mise en contact de parois de cellules de levure avec une solution alcaline aqueuse dans des conditions appropriées pour réaliser l'extraction de protéines et de lipides ;
(b) la séparation des parois de cellules de levure extraites résultantes de ladite solution alcaline aqueuse ;
(c) le lavage des cellules de levure séparées résultantes de manière à en retirer encore des composants des parois de cellules solubilisés ;
(d) la neutralisation des parois de cellules de levure lavées ; et
(e) la pasteurisation des parois de cellules lavées neutralisées puis le séchage des parois de cellules lavées neutralisées pasteurisées résultantes.